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DEPARTMENT OF THE ARMY
Fort Detrick
Frederick, Maryland

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Knowledge about the pigment of Bacillus prodigiosus.

First report: On pigment production.

by Dr. G. Gorbach

From the Institute for Technical Biochemistry and Microbiology of the Technical College at Graz. Zentralblatt f. Bakter. 2. Abt. Vol. 79, No. 1/7, 1929, pp 26-50.

Already in the early stages of bacteriological research it was customary to use pigment production as a factor in the differentiation of bacterial types. It was shown subsequently, however, that pigment production is a quite variable property of certain bacterial types which depends on a number of external conditions, the knowledge of which is by no means exhausted even today. For this reason it was frequently observed that a type with vigorous pigment formation discontinued this activity after a shorter or longer period of cultivation in the laboratory, seemingly without a particular external cause, and then grew without pigment or at any event with reduced chromogenesis and an altered shade of color.

This variability in pigmentation is shown best by Bacillus prodigiosus, probably the most extensively and most frequently examined type of bacterium.

Hefferan (1) has used several strains of prodigiosus in her comparative tests of a number of red bacteria. Agar plate cultures obtained from them showed quite variable amounts of pigment. The number of colored and weakly or not at all colored cultures fluctuated within wide limits for the individual strains.

Hefferan was unable to furnish exhaustive clarification based on unequivocal tests. These examinations confirmed the phenomenon known even then, that B. prodigiosus shows the ability to produce pigment only at low temperatures below 33°C, while this ability is already completely eliminated by temperatures of around 37°C.

It suggests itself to examine the ability to produce pigment with a view to its possible significance to the producing organism. Hereon, too, numerous opinions exist, based on more or less exact tests, without unequivocal solution of these problems. Considering the frequent diversity in the organization of the known bacterial types, a generally valid answer cannot be given. For those types in which optimal growth does not coincide with ximal chromogenesis, no particular value may be ascribed to pigment formation for the life of the organism and the maintenance of its vital properties. The preceding considerations do not allow the assignment of a vital function to the pigment production of B. prodigiosus. As is well known, this bacterium only secretes its red pigment into its surroundings if, among other factors, the growth temperature lies between the usual room temperature and 37°C. If it rises above 37°C, growth is excellent, but no pigment is formed. Therefore the process of chromogenes-

does not seem to be important to the growth of B. prodigiosus.

The great importance of the composition of the nutrient substrate for pigment formation, in addition to the utilized temperature, had been recognized early. Hefferan (l.c.) and Sullivan (2) may be mentioned, who have called special attention to the importance of the knowledge of the medium's composition in pigment production tests. The composition naturally influences the process of metabolism. In addition, various rays seem to be important to pigmentation, especially light, also high pressures, constant passive movement of the culture, the type and quantity of gases dissolved in the fluid, the viscosity of the medium and its reaction.

All investigations conducted to date concerning the pigment production of bacteria in general and of B. prodigiosus in particular, show that it is primarily a function of metabolism and that an insight into the processes of pigment formation will be possible only by way of metabolic tests.

The report on my own tests with nutrients of exactly known composition and of maximal simplicity shall be preceded by the literature relating to the metabolism of B. prodigiosus and the pigment of this type, so far as it pertains to the question of chromogenesis of this bacterial type. In this connection a sifting of the papers according to the following scheme seems indicated:

I. Influence on pigment production

1. By the medium with reference to

a) the source of carbon and nitrogen

b) the existing hydrogen ion concentration and the nutrient salts.

2. By the concentration of oxygen.

3. By physical influences, such as temperature, rays, pressures and jolts.

4. By toxins.

II. Prodigiosin.

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I. Influence on pigment production

1. By the medium with reference to

a) the sources of carbon and nitrogen.

The carbohydrates deserve primary consideration as nitrogen-free carbon sources. The beneficial influence of starch on the pigment formation of B. prodigiosus has been known for some time. Thus a potato medium is able to stimulate those strains of prodigiosus into renewed vigorous pigmentation which have almost or completely lost this ability due to frequent cultivation

on artificial media.

Schneider used starch-containing media in tests of the chemical properties of bacterial pigments. Kraft (3), Pappenhausen (4) and Hefferan (5) also were able to observe the favorable influence of starch on the production of pigment. According to Pappenhausen the amount of pigment even rises in proportion to the starch content of the nutrient.

Opinions differ on the value of the various sugar types for chromogenesis.

Wasserzug (6) thinks that sugar is indispensable to favorable pigment production, Novley (7) and Koeske (8) on the other hand claim to have seen better pigmentation in sugar-free nutrients. Hefferan (9) has closely examined dextrose, lactose and saccharose for their influence on pigment production. She used a synthetic nutrient solution consisting of 0.1% KH_2PO_4, 0.2% asparagine and 0.1% $MgSO_4$. Of the three sugars saccharose and dextrose had increased pigment formation considerably in all cases, lactose only in exceptional cases, since the cultures on peptone-agar media and the tested synthetic nutrients showed only meagre pigmentation with and without lactose. Samkow (10) has tested a number of carbohydrates with a view to their influence on the developmental possibilities of B. prodigiosus on nutrients with different nitrogen sources, without considering the effect on pigment production.

Among the simpler carbon sources, glycerol is described as promoting pigmentation. Sullivan (11) and Carminitti (12) found that chromogenesis actually can be increased proportionally with glycerol. According to Sullivan, a decrease in glycerol content must be compensated by a rise in the amount of asparagine up to 1%, in order to maintain good pigment formation. Aside from organic acids, salts of ammonium of succinic acid, lactic acid and citric acid proved useful; these can be utilized as socalled combined nitrogen sources in view of their nitrogen content. According to Sullivan (11) they yield the best pigment when combined with asparagine.

The salts of ammonium of other organic acids, such as wine vinegar, oxalic acid, uric acid and formic acid are not favorable to pigment production, although growth of B. prodigiosus has taken place.

Hardly any data exist on the influence of nitrogen sources on pigmentation.

Hefferan has determined an inter-dependency between the sources of carbon and nitrogen to the extent that in the absence of particular carbon sources (carbohydrates) the amount of asparagine in the medium had to be doubled before chromogenesis occurred. Sullivan's tests also pointed to the necessity of using suitable combinations of nitrogen compounds with carbohydrates and nutrient salts in the media. Thus he demonstrated that the pigment is most rapidly produced in a solution of 0.2% asparagine, 0.2% $MgSO_4$, 1% K_2HPO_4 and 2% glycerol. Tests of the influence of inorganic nitrogen sources in combination with carbohydrates and other carbon sources on pigment production apparently have not yet been conducted.

b) Hydrogen ion concentration and nutrient salts.

The influence of the pH factor in the nutrient has been treated but in a few experiments, which moreover are of little value owing to the inaccurate determination of acid and alkali contents.

Thus Kuntze (13) found that a wekly acid reaction is favorable to pigment formation, while growth is not furthered thereby. Papenhausen (14) also recognized the favorable effect of weakly acid media on pigmentation, as long as growth is not overly harmed. Laurent (15) has attributed the beneficial effect of carbohydrates to the acids being formed in their degradation, resulting in a reaction favorable to chromogenesis. In his opinion the favorable effect of carbohydrates can be eliminated by corresponding addition of alkali. This view is supported by Hefferan's tests which demonstrated that sugar-free but weakly acidified agar nutrients resulted in as vigorous a pigment formation as saccharated media. In these tests nutrient agar with 1.5% agar and 1% peptone was utilized. Of this, one sample was neutral (indicator phenolphthalein) and one portion each with an acid or alkali content of 1.5% was used. The cultures grown thereon showed the best pigmentation in the acid reaction and the least in the alkaline. In the first case the pigment was vermilion red, in the second violet.

There are extensive studies of the influence of inorganic nutrient salts on pigment production of B. prodigiosus. Most of the investigators agree that magnesium assumes a major role in pigmentation, since its presence seems to be absolutely necessary. This fact may be justly generalized as being true of the pigment production of bacteria and yeasts on the whole.

Thus Pseudomonas pyocyanea does not produce bacteriofluorescein if the nutrient lacks magnesium, as demonstrated by Kuntze (16). Kossowitz (17), in his examinations of pigment-producing saccharomycetes, came to the conclusion that they, too, unquestioningly require the presence of magnesium salts. Samkow's (18) tests with B. prodigiosus produced the significant fact that in this case the utilization of magnesium sulfate in the cultur medium not only makes pigmentation possible, but also promotes growth. This was shown upon cultivation in a nutrient solution containing potassium phosphate and soda and small amounts of magnesium sulfate in addition to asparagine and glycose.

Samkow's experiments show further that the anion connected with magnesium has no significance for pigment production. The tests also determined the necessity for the presence of $PO_4^{\cdot\cdot\cdot}$ and $Cl^{\cdot}$.

Papenhausen (19) and Sullivan (20) also arrive at the conclusion that Mg-cation is indispensable in the medium for pigmentation purposes. With respect to sulfides, iodides and bromides, the authors conclude that these salts hinder rather than further pigment formation.

Smirnow (21) reports that NaCl and Na_2SO_4 also are injurous to pigmentation, which may be true of higher concentrations.

2. Influence of oxygen concentration.

Schottelius (22), to whom we owe the first investigations of the chromogenesis of B. prodigiosus, already found that a surplus of air is necessary

for favorable pigment production of this bacterial type.

Wasserzug (23) observes a lower chromogenesis in liquid cultures of B. prodigiosus than on gels (agar). He attempts to explain this phenomenon by the lower oxygen concentration in the first as compared to that of the air. Galeotti (24) did not find an increase in pigmentation upon utilization of pure oxygen in place of air, leading him to the conclusion that too high an oxygen concentration as well as too little tends to inhibit pigment production. Delanoe (25) tested the effect of aerial oxygen on liquid cultures by increasing the surface of the nutrient by tilting the test tube, and obtained better pigmentation in the tilted tube as compared to the perpendicular tube. Frequent laying down and setting up of the tubes also favored pigment formation, since the liquid medium is more thoroughly aerated. However, these tests do not make it clear whether the increased oxygen content only promotes growth and thus indirectly furthers pigmentation, or only promotes the latter without regard to multiplication. The fact that a certain oxygen pressure must exist for favorable growth was demonstrated by means of anaerobic cultures of Bacillus prodigiosus, which according to Delanoe show only meagre growth.

Ritter claims to have proved that B. prodigiosus also is able to grow under strict anaerobic conditions. Samkow (27) contends that upon reliable elimination of aerial oxygen B. prodigiosus is unable to maintain growth, agreeing with Delanoe's findings.

3. Influence of physical forces.

As already stated, the growth temperature exerts conspicuous influence on the pigmentation of B. prodigiosus.

Schottelius (28) first called attention to this phenomenon. He noted that this type of bacterium is unable to produce its pigment at 37°C in spite of lush growth. Upon constant cultivation at this temperature, favorable to reproduction, chronic damage to the ability to form pigment ensues, which is not easily removed even by subsequent cultivation at room temperature and at 28°C. Strains of B. prodigiosus develop whose growth form is without pigment. Delanoe (29) sets the optimal temperature for pigmentation at 17-23°C and gets the same results as Schottelius with respect to the effects of higher temperatures. He finds 43°C to be the maximum temperature for growth.

Opinions differ concerning the effect of visible and ultraviolet rays on chromogenesis. The cause thereof is frequently found in the methods utilized. Since prodigiosin as such is very photolabile, exposure tests may not be conducted with extended periods of irradiation. Long periods of exposure lead to the bleaching of the pigment, simulating an absence of pigment production. Besides, extended irradiation injures growth and may inhibit it altogether, thus hindering pigmentation indirectly.

The tests of Galeotti (30), Hefferan (31) and others with extended direct exposure to sunlight result in a decrease of pigment formation in cultures of B. prodigiosus, but this is coupled with inhibition of its growth. Therefore a direct influence of sunlight on pigmentation cannot be deduced from these tests.

Earlier investigations reveal that growth and pigment production are not damaged by red and yellow as well as heat rays during irradiation with visible or ultraviolet light, but solely by the shorter wave length of the visible spectrum and ultraviolet rays as such. Generally a 48-hour exposure suffices for suppression of pigment formation and enzyme production. Gottschlich (32) claims to have demonstrated that short exposures to violet or ultraviolet rays exert a beneficial influence on growth and chromogenesis. He explains his findings by contending that the less resistent and old cells are annihilated by short exposure to sunlight, while the viable young forms survive and are stimulated into particularly vigorous cell division and pigment formation. More detailed investigations concerning the effect of light on the pigmentation of B. prodigiosus, based on modern methods of measurement, are lacking.

Chopin and Tamann (33) investigated the effect of high pressures on pigment production, concluding that about 3,000 atmospheres inhibit development of B. prodigiosus, consequently also pigmentation. Initially they noted a slowing down of the cellular motility. As yet there are no important investigations of other physical influences.

4. Influence of toxins.

This field also shows few investigations. Smirnow (34) claims to have influenced pigmentation favorably with phenol fumes directed on agar cultures of B. prodigiosus. This increased pigment production is said to persist for several generations. Cordier (35) exposed cultures to ether fumes and was able to thus suppress chromogenesis. Subsequent generations are also said to have grown achromogenically for some time. He obtained the same results with fumes of methyl and ethyl alcohol and chloroform.

As a supplement, the effect of radium emanation on pigmentation shall be touched on briefly, as claimed by Bouchard and Baltnazar (36). According to these authors, radium emanations initially exert an unfavorable influence on the growth of all chromogenic bacteria which secrete pigment into their surroundings, without hindering pigment production to an important degree.

II. Prodigiosin.

Schneider (37) made the first attempt to examine the glowing, blood-red pigment of B. prodigiosus, "prodigiosin," with respect to its physical and chemical properties. He noted the solubility of this bacterial pigment in alcohol, ether, carbon disulfide, chloroform and glacial acetic acid, and its complete insolubility in water. No alteration of the pigment occurred in the above solvents. The alcoholic solution shows a carmine red hue upon admixture of hydrochloric acid, shifting into red-violet after a short time. The prodigiosin solution is stained brownish-yellow by potash lye and ammonia. Careful addition of hydrochloric acid to the stained solution resulted in a return to the red-violet hue. Chlorine water removes the color via reddish-brown and golden yellow. Dry prodigiosin obtained from the solution by evaporation of alcohol shows a brown color after moistening with sulphuric acid. Exposure to sunlight of prodigiosin solution causes complete bleaching within a relatively short time.

According to Schneider the absorption spectrum of prodigiosin shows a strong darkening from 66-70 millimicrons and above. Violet and blue disappear completely.

Few reports exist on the chemistry and constitution of prodigiosin; they utterly fail to solve these problems. They are mentioned here only for the sake of completeness. Griffiths (38) assigns the elementary formula

$$C_{38}H_{56}NO_5$$

to prodigiosin, corresponding to a nitrogen content of 2.3%.

Kraft (39) on the other hand reports a considerably higher nitrogen content allegedly reaching 3.9%, while Scheurlen (40) even declares prodigiosin to be nitrogen-free, a conclusion recently confirmed (41).

Samkow (42) expected prodigiosin to contain bound magnesium, reasoning from the premise that this element is of decisive importance for pigment production. Despite the use of very sensitive methods he did not succeed in demonstrating magnesium in the ash of prodigiosin.

Prodigiosin's sensitivity to acid and alkali led Kraft (l.c.) to the presumption that this pigment is a "dye acid" which is transformed into the corresponding dye salt in the presence of free alkalis. This is supported by the observation that the alkali salt of prodigiosin is in fact water soluble.

Eisler and Portheim (43) report that prodigiosin is temperature-sensitive in an alcoholic solution. If the orange-red prodigiosus extract produced in 70% alcohol is boiled, its color shifts to reddish-violet. Cooling causes it to return to the original color. This reversible process may be repeated several times. Finally the solution remains reddish-violet. Admixture of distilled water without boiling resulted in the same phenomenon of color-transformation. The authors attributed this action to the water content of the solvent, which determines the size of the particles and thus the tint.

III. Test methods.

a) Production of the mineral nutrient.

All tests discussed in this paper utilized a mineral nutrient solution, the composition of which generally resembles the nutrient of Arthur Meyer supplemented with the addition of lithium chloride, as many investigations ascribe a favorable influence on chromogenesis to the latter.

Arthur Meyer's nutrient solution contains the following: 1 g KH_2PO_4, 0.1 g $CaCl_2$, 0.3 g $MgSO_4 + 7\ H_2O$, 0.1 g NaCl per liter. To these salts LiCl is added in the same amount as magnesium sulfate.

The primary KH_2PO_4 in this form is not suitable for the maintenance of acid or alkali concentration, even when diluted, but it is satisfactory combined with secondary K_2HPO_4. As a tribasic, weak acid, phosphoric acid in conjunction with strong bases can act as buffer and is able to regulate the H ion concentra-

tion, i.e. the ratio of dissociated and undissociated acids, upon dilution. For this reason the content of primary phosphate was partly replaced by secondary phosphate. In this manner the desired pH concentration of the nutrient is achieved and maintained.

In order to satisfy the osmotic requirements, the nutrient was combined not by grams, but by gram-moles or millimoles.

According to these aspects, 1,000 ccm of prepared, usable nutrient solution contain the following salts in grams:

KH_2PO_4: K_2HPO_4 in changing amounts according to the desired pH factor, corresponding in toto to 1/100 mole of primary potassium phosphate:

KH_2PO_4	1.362 g	equals	1/100 mole
$CaCl_2$	0.111 g	"	1/1000 "
$MgSO_4$	0.3606 g	"	3/1000 "
NaCl	0.117 g	"	2/1000 "
LiCl	0.127 g	"	3/1000 "

Upon dilution of these salts in distilled water, turbidness ensues. This is due to the insolubility of secondary $CaHPO_4$ being formed in the solution. Turbidness intensifies if the fraction of secondary phosphate must be increased in order to obtain a certain pH value. The amount of $CaCl_2$ was therefore reduced to 1/10, so that only 0.011 g CaCl2 is used per 1,000 ccm of solution. Growth is not unfavorably influenced thereby, as shown in preliminary tests.

In the production of the solution, above listed salt ratios of the final nutrient were derived from concentrated standard solutions in the form of normal solutions of the salts, in order not to reduce the final salt content by addition of the necessary carbon and nitrogen sources and the adjustment of the planned H ion concentration.

For the production of 100 ccm of neutral nutrient solution the following quantities of normal salt solutions are required:

10 ccm of a mixture of 2 1/10: 1 1/10 NK_2PHO_4 and KH_2PO_4 (about 2:1)
3 ccm.....of 1/10 N $MgSO_4$
2 ccm.....of 1/10 N NaCl
3 ccm.....of 1/10 N LiCl
1 ccm.....of 1/100 N CaCl
19 ccm total

81 ccm remain for other additions.

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Adjustment of pH concentration in synthetic and natural media.

General adjustment of a certain pH value may be achieved with the aid of the phosphate mixture. Corresponding ratios of the mixture were obtained by A. Fodor's method (44). For neutral media this ratio amounts to 2 parts secondary and 1 part primary phosphate. For the adjustment of the different pH values in a test series for the study of the effect of pH values as such,

a corresponding amount of 1/10-N-acid and alkali, respectively, was used. The necessary quantity for gradual increase of the pH value was determined with sufficient precision with the aid of the colorimetric method after Michaelis (45) for colored and turbid liquids.

Production of nutrient solutions in concentration tests.

In concentration tests the media were produced somewhat differently, to the extent that a doubly concentrated standard nutrient solution was used as a starter. This made a twofold number of ccm available for addition of dissolved special C and N sources without alteration of the final saline concentration.

The scheme of such tests was the following:

10 ccm of mineral nutrient solution (doubly concentrated) + number of ccm of N or C source constant + x ccm N or C source variable + ccm 1/10 N-acid or alkali for the adjustment of desired pH value + ccm distilled water for filling to 20 ccm.

Tests covered an area of concentration of 0.1-10%, in some cases 0.1-1%. The adjustment of such a concentration series proceeded very simply according to the following scheme:

1. One 2% and one 20% solution of the C and N source, respectively, are prepared. The solution becomes 0.1% and 1%, respectively, upon dilution to the twofold amount.

2. The constant amount of the C or N source is dissolved in the concentrated mineral nutrient solution. The adjustment of pH is not necessary here, since the entire series must show the same pH value which does not change in neutral nutrients and well-buffered nutrient solutions.

Individually, the scheme is the following with a constant N source and a variable C source:

No. 1: 10 ccm (doubly concentrated mineral nutrient solution + g of constant N source (NH_4Cl, asparagine)) + 1 ccm of a 2% solution of the carbon source (glycerol, sugar) + 9 ccm distilled H_2O.

No. 2: 10 ccm (doubly concentrated mineral nutrient solution + g of constant N source (NH_4Cl, asparagine)) + 2 ccm of a 2% solution of the carbon source (glycerol, sugar) + 8 ccm distilled H_2O etc.

Three test tubes were then filled with exactly 6 ccm nutrient solution and labeled with the pertinent number. After corresponding sterilization in the autoclave (3 x 20 minutes on three consecutive days) and standing for 4 days to test sterility, transplanting could be started. Each concentration series of 0.1-10% therefore contained 60 test tubes. By three times conducted testing a result was achieved which sufficiently well guaranteed the elimination of chance.

Test series dealing with pH concentration.

In these test series the mineral nutrient solution, the carbon source and the nitrogen source remained constant. Only the pH concentration was gradually changed by 0.2. The adjustment of these values was done by addition of a corresponding amount of 1/10 N-acid or alkali. Values were adjusted colorimetrically. After threefold sterilization in the autoclave the constancy was tested. If the nutrient solution had remained sterile, transplantation could begin after four days.

b) Method of transplantation.

In order to transfer nearly the same amount of bacteria into the individual tubes within the comparative series, inoculation took place from the synthetic nutrient solution proper or from suspensions of a certain amount of agar culture in 0.75% NaCl solution with an invariable and deeply introduced platinum wire loop.

c) Determination of chromogenesis.

Tests without exact measurement of pigment may be highly misleading. Due to the lack of color of B. prodigiosus proper, poor pigment production may be simulated during vigorous growth, since the red pigment may be covered by the white bacterial mass, and the culture appears a pale pink. On the other hand, the pigment may come into greater prominence with poor growth and may simulate more vigorous pigmentation, although it is actually scanty.

A gravimetric measurement of pigment cannot be made in view of the relatively small yield of pigment. One is limited to the extraction of pigment from equal amounts of a solvent and colorimetric, comparative determination of increase or decrease in pigment, applied to the strength of the color. If the solution showing the strongest color is used as a yardstick, useful values can be obtained within a test series. The extraction of the pigment of B. prodigiosus is not difficult in view of its insolubility in water and solubility in ether, alcohol etc. The various liquid cultures of a test series are simply extracted in the separatory funnel with a solvent immiscible with water until colorless. In the following tests ether was utilized which absorbs pigment with particular rapidity and ease. The ethereal solution is initially blood-red, but loses its color after a short period of standing. For this reason the colorimetry of ether solutions offers difficulties, other than the change in concentration and rapid evaporation of the ether. The ether solution is therefore evaporated on the electric water bath and the remaining pigment is absorbed by 96% alcohol. The quantity of alcohol amounted to 5 ccm for each residue. Upon shaking of the culture liquid with ether, emulsions were easily formed which could be avoided by a surplus of ether. The separation of ether from the culture liquid was promoted by centrifugation.

d) Determination of growth.

The counting of cells appearing after a certain time in the liquid culture was chosen for the determination of growth. Counting was carried out by means of Thoma's counting chamber.

Most cultures showed strong formation of zoogloea after 8-14 days, which must be dispersed by shaking prior to the count. From the vigorously shaken culture a uniform quantity of suspension was withdrawn by means of an invariably identical platinum wire loop, transferred to the counting chamber and usually diluted with one drop (for dense bacterial aggregates with two drops) of diluted methylene blue solution (1 ccm concentrated methylene blue solution + 9 ccm distilled H_2O). The mixing of the drop of culture with methylene blue must be accomplished rapidly by delicate rubbing with the loop on the counting surface, in order to minimize evaporation. In order to achieve rapid staining and inactivation of the motile bacteria, the methylene blue solution contains a few drops of formol. Counting was done with eyepiece 3 and lens E (Zeiss). From the 400 available counting areas, 60 were counted, with particular attention to border squares and diagonal fields. The arithmetic medium was established from the total of obtained figures and the average number for the field was thus determined.

e) Determination of the final pH value.

The determination of the final pH value, i.e. the hydrogen ion concentration at the end of the test culture was conducted colorimetrically after Michaelis. It was established in these tests that the pH values are not changed by an extraction of the cultures with neutral sulfuric ether.

IV. Morphological and biological properties of the utilized prodigiosus culture.

The culture of B. prodigiosus utilized in the tests came from the collection of the Hygienic Institute of Graz University. It was sent on a gelatinous plate.

The utilized nutrient gel had the following composition:

½% peptone 1% dextrose 1% NaCl 10% gelatin

Neutralization (indicator phenolphthalein) - albumen clarification. pH equals 7.6 after colorimetric determination.

Within 24 hours at 20°C, delicate and colorless colonies develop on the plates, of which the superficial ones are surrounded by a narrow circle of liquefaction. After 48 hours the surface colonies have increased to 1 mm in diameter. They lie in deep, dish-like funnels of liquefaction. Their microscopic and macroscopic habits agree precisely with the description in Lehmann and Neumann's Bacteriology (l.c.).

A hanging drop prepared from the gelatinous colonies shows briskly motile, short and plump rods. A smear preparation of a 48-hour culture stained with methylene blue or diluted fuchsin solution contains only uniformly and intensely stained rod forms. The cells are gram negative.

Chromogenesis appears in the cultures after about 48 hours at 20°C. At this time the deposit is colored a pale pink.

Gelatin stab cultures were started on various acid, neutral and alkaline

nutrient gels of above-stated composition. In order to achieve as nearly an equal inoculation of the individual tubes as possible, the inoculation was accomplished with a platinum needle which was dipped to a uniform depth into the evenly stirred bacterial suspension. Table 1 contains the results.

Tab.1. Growth of B. prodigiosus in gelatin stab cultures.

Days at 20°C	growth manifestations	pH 5.4	pH 6.2	pH 7.0	pH 7.6	pH 8.4
3	liquefaction	weak	funnel 3 mm	funnel 5 mm	funnel 3 mm	funnel 3 mm
	pigmentation	-	-	weak pink	-	-
	gasification	-	-	scanty	strong	strong
6	liquefaction	funnel 2 mm	funnel 10 mm	entire width	entire width	funnel 10 mm
	pigmentation	weak pink	good	good	good	-
	gasification	-	-	scanty	strong	strong
8	liquefaction	funnel 4 mm	entire width	complete	entire width	entire width
	pigmentation	good	very good	very good	good	weak
	gasification	-	-	scanty	strong	strong
12	liquefaction	entire width	entire width	complete	entire width	entire width
	pigmentation	very good	very good	very good	good	weak
	gasification	-	-	scanty	strong	strong

Agar streak cultures with reactions corresponding to pH values 5,6,7 and 8.2 at growth temperatures of 20°, 28° and 37°C show the behavior compiled in table 2 with respect to growth and pigment production.

The utilized nutrient agar had the following composition:

Meat broth (1 kg horse meat / 2 l water).....1000
peptone sicc.....10 glycerol.....20
sodium chloride.....5 agar.....20

The following nutrient served in the preparation of agar streak cultures:

Meat broth (1 kg horse meat / 2 l water).....1000
peptone sicc.....10 glycerol.....30
dextrose.....5 sodium chloride.....5

The reaction corresponded to pH 7.5 by colorimetric determination. Cultivation took place at 28°C. In the first 24 hours a weak growth was seen along the entire inoculating stab, which rapidly increased at the surface. Initially white deposits around the stab turned more intensely pink from the

Tab.2. Growth of B. prodigiosus on agar streak cultures.

Growth in days	pH 5.6 20°C	pH 5.6 28°C	pH 5.6 37°C	pH 7 20°C	pH 7 28°C	pH 7 37°C	pH 8.2 20°C	pH 8.2 28°C	pH 8.2 37°C
1	growth along streak just visible	weak growth, weak pink color	trace of growth, no pigment	barely visible growth, no pigment	very good growth, weak pink color	good colorless growth	very scanty growth, no pigment	good growth, weak pink color	weak growth, colorless
2	weak growth, pink color	good growth, light red pigment	white, ca.2mm wide deposit along streak	good growth, good pigmentation	especially good growth, strongly light red	very good growth, colorless	scanty growth, weak pink color	better growth, light pink color	scanty growth, colorless
3	good growth, blood red color	good growth, strong pigmentation	strong growth, colorless	very good growth, very good pigmentation	especially good growth, good pigmentation	excellent growth, colorless	good growth, pink color	well grown, light pink color	moderate growth, colorless
4	good growth, very good pigmentation	very good growth, strongest pigmentation	very strong growth, colorless	very good growth, much blood-red pigment	especially good growth, strong red color	excellent growth, colorless	very good growth, pink color	well grown, light red color	good growth, colorless

second day of growth. After 3 days the nutrient is permeated with gas bubbles.

For comparison of growth manifestations of the utilized strain of B. prodigiosus, broth cultures were prepared and maintained at 20°C. The nutrient broth had the following components:

Horse meat broth	1000	Dextrose	5
peptone sicc	10	sodium chloride	5

Reaction weakly alkaline, corresponding to pH 7.4.

In the first 24 hours a slight turbidness appeared, which increased in the following days and after 10 days settled in part to the bottom. After 5 days the first traces of redness appeared on the surface; later the precipitated flakes were stained red. In older cultures, formation of zoogloea was particularly noticeable.

Cultivation tests of B. prodigiosus in the Buchner tube closed with a rubber stopper led to the assumption that some growth also occurs under strongly reduced oxygen pressure, but that chromogenesis fails to take place under these conditions.

V. Pigment production upon cultivation with a constant N source and a variable carbon source.

Concerning these tests it should be noted that a number of preliminary tests of the effect of various carbon sources were conducted, in order to gain a certain perspective on the influence on chromogenesis of commonly used carbohydrates, such as starch, dextrine, cane sugar, lactose, maltose, galactose, dextrose and levulose, and the polyvalent alcohols mannite and glycerol. Subsequently dextrose was singled out as simple sugar and trivalent glycerol as lower alcohol, and these two carbon sources in conjunction with asparagine and ammonium chloride as constant nitrogen sources were subjected to calibrated test series. In all series the concentrated mineral nutrient solution described in the foregoing chapter served as a basis for the test medium; the addition of carbohydrates or alcohols and the uniform adjustment of the pH value proceeded according to the method already discussed. A two-day agar culture grown at 28°C, from the surface film of which a suspension was prepared, served as starting material for all inoculations. The tests usually lasted 14 days.

In the following, the test series will be discussed briefly.

Test series 1.

This was conducted with a quantity of 0.5% asparagine as special nitrogen source and with 1.5% of various carbohydrates and alcohols in the mineral solution with acid and alkaline reactions. The amounts of pigment forming in the culture tubes after varying lengths of time were estimated comparatively. Quantities of pigment developing after 14 days of cultivation were measured, after extraction from the culture, with Duboscq's micro-colorimeter in the manner described in the preceding chapter.

Tab.3. Influence of C sources (+ asparagine) and reaction on the chromogenesis of B. prodigiosus.

C-source 1.5%	pH 6.6 growth in days				pH 7.5 growth in days			
	3	7	9	14	3	7	9	14
starch (soluble)	+	++	++	++	+	+	+	+
dextrine	+	++	++	++	-	-	+	+
cane sugar	+	+	+	+	+	+	++	++
lactose	-	+	+	+	-	-	-	-
maltose	+	+	+	+	+	+	+	+
galactose	+	++	+++	+++	+	+	+	+
dextrose	+	+	+	+	+	+	+	+
levulose	-	+	+	+	-	+	+	+
mannite	-	+	+	+	-	-	-	-
glycerol	++	++	+++	++++	+	++	+++	++++

Table 3 reveals that pigment formation is somewhat retarded by an acid reaction, but that hardly any difference in chromogenesis is caused by the reaction after 14 days. It is evident further that, next to starch and dextrine, galactose and glycerol may be considered particularly favorable sources of carbon.

If the colorimetric results are expressed in percent of pigment quantity of the most strongly colored culture, the following values are obtained at the end of 14 days of testing, utilizing pH 6.6 and 7.5 as starting reactions:

C-source	Initial pH 6.6 %	Initial pH 7.5 %	C-source	Initial pH 6.6 %	Initial pH 7.5 %
I. starch	35.0	32.5	VI. dextrose	8.8	12.5
II. dextrine	47.5	37.5	VII. levulose	87.5	100.0
III. saccharose	12.5	9.0	VIII. galactose	62.5	62.5
IV. lactose	42.5	10.0	IX. mannite	20.0	27.5
V. maltose	100.0	67.5	X. glycerol	62.5	60.0

At first glance, disagreement with pigment estimation is noted in this test series. It is, as has been explained previously, due to the fact that the amount of pigment appears hidden in the presence of vigorous growth, and no direct relationship exists between the speed of multiplication and the quantity of pigment formed.

Percentages of pigmentation may also be interpreted to mean that the initial reaction of the medium between pH 6.6 and 7.5 has no significance or little significance for the promoting or inhibiting effect of certain C sources such as starch, dextrine, saccharose, dextrose, galactose, mannite and glycerol, while the effect of the reaction is quite great for lactose, maltose and levulose.

Test series 2.

Since asparagine is a carbonaceous source of nitrogen, it was interesting to resort to a carbon-free compound as nitrogen source for the testing of the effect on chromogenesis of various carbon sources. Ammonium chloride proved useful for this purpose in quantities of 0.5%. However, B. prodigiosus does not grow too well in the presence of this inorganic nitrogen source, which is the reason why chromogenesis, if such occurs at all, can be tolerably estimated and measured only from the fourth day of cultivation on.

The preparation of the nutrient for this test series conformed to the first. Reactive conditions were changed only to the extent that pH 5.4, 7 and 7.4 were chosen as initial reactions of the medium. Inoculation and cultivation were conducted under conditions identical with the first series.

Tab.4. Influence of C sources (+ NH_4Cl) and reaction on the chromogenesis of B. prodigiosus.

C-source 1.5%	Initial pH 5.4 Growth in days			Initial pH 7.0 Growth in days			Initial pH 7.4 Growth in days		
	4	8	14	4	8	14	4	8	14
starch	+	+	+	+	+	+	-	+	++
dextrin	-	-	+	-	+	+	-	+	+
saccharose	-	-	-	+	+	+	-	+	+
lactose	-	-	+	-	-	+	-	-	-
maltose	-	-	+	+	+	+	+	+	+
galactose	-	-	-	+	+	+	+	+	+
dextrose	trace	+	+	+	+	+	+	+	+
levulose	-	-	-	-	+	+	-	-	-
mannite	-	-	-	+	+	+	+	+	+
glycerol	+	+	+	+	+	+	+	+	+

Table 4 shows that, generally speaking, pigment production is much lower than with asparagine as nitrogen source. Growth also is scanty. It is clearly shown, moreover, that the acid initial reaction hinders pigmentation most during the first four days; the alkaline reaction less so. A neutral reaction of the medium seems best. Later the effects of originally neutral and alkaline reactions nearly balance out, while hindrance by the acid starting reaction persists.

If the amounts of pigment produced after 14 days of cultivation are determined colorimetrically and again are applied percentually to the maximum of pigment obtained (100%), values compiled in table 5 result.

Table 5

Carbon source	pH 5.4 %	pH 7.0 %	pH 7.4 %
I. starch	5.0	12.5	100.0
II. dextrin	9.0	18.7	12.5
III. saccharose	0.0	4.0	4.0
IV. lactose	25.0	18.7	3.8
V. maltose	9.0	30.0	30.0
VI. galactose	0.0	5.0	10.0
VII. dextrose	2.5	4.0	3.8
VIII. levulose	0.0	5.0	3.8
IX. mannite	0.0	15.0	3.8
X. glycerol	7.5	30.0	35 .0

The important result of the first two test series is the conclusion that different carbon sources as well as variable nitrogen sources are significant for chromogenesis at particular pH values. The question presented itself, to which extent pigment production is influenced by the concentration of the utilized carbon source with a particular reaction of the nutrient solution, in the presence of a constant carbon-free and carbonaceous source of nitrogen, and what kind of changes the adjusted initial hydrogen ion concentration reveals after the termination of the test. At the same time the size of cell multiplication and the relationship between chromogenesis and growth and multiplication, respectively, was investigated. All results compiled are the mean of three identical parallel tests. The method of pigment evaluation by means of colorimetry was retained, likewise the technique of serial dilution of the nutrient solution. An attempt has been made to express the extent of multiplication numerically by fixing the average number of bacteria in all counted squares for each tested tube as the size of increase.

Test series 3.

The mineral nutrient solution mentioned at the beginning contained, beside the described salts, 0.535% ammonium chloride as N source and glycerol in ascending quantities from 0.1 to 10% as C source. The initial reaction of the nutrient solution corresponded to pH 6.8. Cultivation took place at 28°C. An attempt was made to transplant an identical amount of B. prodigiosus from the suspension of a 48-hour agar culture to the individual tubes by means of a platinum needle which was invariably submerged to the same depth. The result of this test series after 14 days is compiled in table 6, in which the pigment values signify percent of the deepest color obtained, and values of multiplication refer to the average number of bacteria applied to 1 field.

The peculiar and seemingly unmotivated jumps at 0.4, 0.7-0.9 and at 2% glycerol are conspicuous. We should be dealing primarily with errors in inoculation. Still, the contradiction between reproduction and chromogenesis at 2 and between 3 and 6% cannot be explained adequately.

Tab.6. Pigment production and cell multiplication at different glycerol concentrations (+ NH_4Cl).

Glycerol %	Pigment %	Extent of multiplication	Glycerol %	Pigment %	Extent of multiplication
0.1	2.5	14	2.0	20.0	13.0
0.2	2.5	12	3.0	100.0	15.0
0.3	12.5	10	4.0	100.0	11.5
0.4	2.5	8	5.0	27.5	11.0
0.5	27.5	12	6.0	22.5	19.0
0.6	17.5	7	7.0	15.0	19.0
0.7	2.5	6.5	8.0	7.5	13.0
0.8	2.5	7.0	9.0	2.0	2.5
0.9	2.5	8.0	10.0	0.0	2.0
1.0	35.0	10.0			

It is certain that, firstly, optimal pigment production took place at a glycerol content of 3-4% and best growth at 6-7% glycerol in the presence of ammonium chloride as nitrogen source and a pH value of 6.8, and, secondly, that under the given conditions the optima of multiplication and pigmentation do not coincide.

Test series 4.

In this series the ammonium chloride of series 3 was replaced by asparagine in the amount of 0.659 g to 100 ccm of nutrient solution, while all other conditions remained the same. Table 7 contains test results.

Tab.7. Chromogenesis and cell multiplication at different glycerol concentrations (+ asparagine).

Glycerol %	Pigment %	Extent of multiplication	Glycerol %	pigment %	Extent of multiplication
0.1	12.5	14	2.0	7.5	52
0.2	25.0	20	3.0	7.5	16
0.3	100.0	20	4.0	7.5	70
0.4	2.5	14	5.0	10.0	45
0.5	2.5	14	6.0	12.0	32
0.6	75.0	74	7.0	2.5	16
0.7	50.0	12	8.0	2.5	8
0.8	95.0	68	9.0	2.5	8
0.9	55.0	56	10.0	2.5	8
1.0	10.0	58			

It is evident that in the presence of asparagine as N source maximal pigment production (contrary to the findings regarding ammonium chloride) occurs in the area of lower glycerol contentrations and that amounts of over 1% already exert a strong inhibiting influence.

It is shown, moreover, with complete clarity that no constant relationship exists between growth and pigmentation. It is precisely at maximal chromogenesis (0.3% glycerol) that the number of bacteria is very small, while in other cases it almost appears as if the optima of growth and pigmentation coincided. One could hardly go wrong in the assumption that the reproduction of B. prodigiosus is good at 0.6 to about 4% glycerol in the presence of asparagine and that it rapidly falls off at lower or higher glycerol concentrations.

Test series 5.

This test series was conducted with increasing amounts of dextrose with a constantly maintained content of asparagine as nitrogen source in the mineral nutrient solution. Other conditions were identical to those of test series 4. Already after 5 days all tubes showed good growth with average pigmentation, which later increased. The test was terminated after 14 days and the growth factor, percentual amount of pigmentation and the final pH value were determined. Table 8 lists the results obtained. It shows the dependency of chromogenesis on growth; both functions have their maximum at 0.5% dextrose content in the presence of asparagine as nitrogen source and an initial pH factor of 6.8. If the initial pH values are compared with those of the 14-day old culture, an increase in alkaline reaction up to a content of 0.3% dextrose is evident, while starting at about 0.6-0.7% dextrose and upwards the reaction becomes acid. In the tubes with 0.4-0.6% dextrose the initial reaction of pH 6.8 is approximately retained. In the acid as well as the alkaline areas, growth and especially pigment production is unfavorably affected. Below 0.3%, dextrose does not seem to be utilized, which is the reason for the alkaline reaction occurring at the expense of asparagine cleavage products. Conversely, a dextrose content above 0.7% causes a rapid utilization of this sugar, whose acid decomposition products outweigh the alkaline products of asparagine. At dextrose levels of ½% the reaction products of carbon and nitrogen sources seemed to form neutral compounds. Under these conditions the best growth and optimal pigmentation take place.

Tab.8. Chromogenesis and cell reproduction at various dextrose concentrations (+ asparagine)

Dextrose %	Multiplication factor	pigment %	Initial pH	Final pH
0.1	9.0	5.0	6.8	7.8
0.2	7.0	2.5	6.8	8.2
0.3	15.0	18.0	6.8	7.5
0.4	12.5	52.0	6.8	7.0
0.5	16.0	100.0	6.8	7.0
0.6	12.0	30.0	6.8	6.9
0.7	18.0	30.0	6.8	6.9
0.8	10.0	64.0	6.8	6.8
0.9	11.0	36.0	6.8	6.8
1.0	6.0	2.0	6.8	6.2

VI. Pigment production and growth with constant C and variable N sources.

The following test series were conducted in order to determine how carbonaceous nitrogen sources act on growth and pigment production without addition of a special usable carbon source, and what significance can be ascribed to the concentration of glycerol and dextrose in the presence of a constant, simpler nitrogen source.

Test series 1.

The following combined, i.e. carbonaceous nitrogen sources were chosen for the determination of the influence of various organic nitrogen compounds on pigment formation and reproduction: Peptone, nucleic acid of yeast, alanine, glycocoll, asparagine, sodium of aspartic acid, uric acid, urea and the ammonium salts of tartaric acid, malic acid, lactic acid and citric acid. The basis for the nutrients was again furnished by the mineral nutrient solution to which 3/4% of each of the above nitrogen compounds (with the exception of uric acid) were added, evenly diluted. Due to its poor solubility, uric acid was utilized in concentrated solution. The initial reaction of the medium was nearly neutral, corresponding to pH 6.8, achieved by admixture of diluted hydrochloric acid or soda lye. The test lasted 8 days at 28°C, with an interpolated observation after a growth of 3 days. Table 9 reflects the results obtained.

Tab.9. Pigmentation and cell multiplication in the presence of carbonaceous nitrogen sources.

N-source 0.75%		Duration of growth 3 days	8 days
peptone	pigmentation	-	pale red
	multiplication	weakly turbid	very good
nucleic acid of yeast	pigmentation	-	-
	multiplication	-	-
alanine	pigmentation	-	trace of pink
	multiplication	-	weakly turbid
glycocoll	pigmentation	-	-
	multiplication	-	-
asparagine	pigmentation	weakly colored	weak
	multiplication	weakly turbid	turbid
sodium of aspartic acid	pigmentation	good pink color	good
	multiplication	good	moderate
uric acid	pigmentation	-	weak
	multiplication	-	weakly turbid
urea	pigmentation	-	-
	multiplication	-	-
ammonium of tartaric acid	pigmentation	-	-
	multiplication	-	-
ammonium of malic acid	pigmentation	-	-
	multiplication	strongly turbid	strongly turbid
ammonium of lactic acid	pigmentation	weakly pink	good
	multiplication	good	very good
ammonium of citric acid	pigmentation	-	trace of pink
	multiplication	good	strongly turbid

Table 9 shows that, while generally speaking, the cultivation of B. prodigisus on different nitrogen sources without addition of a special carbon source results in very good growth, pigment production is insignificant. Thus the peptone nutrient solution offers a very good medium for reproduction, but does not promote chromogenesis. On the other hand, sodium of aspartic acid furthers pigment production, without, however, favorably influencing growth. The tabulation also shows that relatively few nitrogen sources promote pigment formation in the absence of a special carbonaceous nutrient.

Test series 2.

In opposition to test 1, this series utilized dextrose in a concentration of 0.5% as carbon source and the number of nitrogen sources was expanded to include the carbon-free nitrogen compounds potassium nitrate and ammonium chloride in amounts of 0.75%. Other conditions of cultivation and nutrition remained the same.

Tab.10. Chromogenesis and cell multiplication in the presence of various N sources (+ dextrose).

N-source 0.75%		Duration of growth 3 days	8 days
peptone	pigmentation	-	weak
	multiplication	strong	strong
nucleic acid of yeast	pigmentation	-	-
	multiplication	weakly turbid	weakly turbid
alanine	pigmentation	-	-
	multiplication	-	weakly turbid
glycocoll	pigmentation	-	-
	multiplication	-	-
asparagine	pigmentation	good	very good
	multiplication	strong	strong
uric acid	pigmentation	trace of red	weak
	multiplication	weakly turbid	weakly turbid
NH_4Cl	pigmentation	-	-
	multiplication	weakly turbid	weakly turbid
KNO_3	pigmentation	-	-
	multiplication	weakly turbid	weakly turbid

Table 10 shows that in some cases the addition of dextrose results in weak growth which is absent when only a combined nitrogen source is used. Dextrose also furthers growth generally and in the case of asparagine, also promotes pigment formation.

The extent to which dextrose (0.5%) and glycerol (1.5%), in conjunction with ammonium compounds, are able to influence growth and pigmentation, is shown by

Test series 3.

Here the standard mineral nutrient solution was mixed with the recorded quantities of dextrose or glycerol and added to 0.75% of the organic ammonium

Tab.11. Pigmentation and cell reproduction in the presence of various organic ammonium salts / dextrose or glycerol

N-source 0.75%	dex-trose	growth after 5 days	growth after 12 days	pigment after 5 days	pigment after 12 days	glycerol	growth after 5 days	growth after 12 days	pigment after days	pigment after days
ammonium of lactic acid	0.5%	very good	very good	good	very good	1.5%	very good	very good	very strong	blood red
ammonium of tartaric acid	0.5%	good	-	-	good	1.5%	good	good	very good	very good
ammonium of malic acid	0.5%	good	-	good	weak	1.5%	very good	weak	very good	weak
ammonium of citric acid	0.5%	good	weak	good	weak	1.5%	very good	weak	good	weak

salts as combined nitrogen source. The initial reaction amounted to pH 6.8. The growth temperature was 28°C. Growth and chromogenesis were estimated after 5 and 10 days.

The test series tabulated in table 11 again reveals that ammonium of lactic acid is best suited for cultivation and pigment production of Bacillus prodigiosus, especially with glycerol as carbon source. It is shown further that in the presence of less easily assimilated nitrogen sources, such as ammonium of tartaric acid, malic acid and citric acid, some success is achieved with glycerol as C source, while dextrose shows varying degrees of failure.

Test series 4.

This series was designed to determine the influence on growth and pigmentation of various concentrations of ammonium chloride as inorganic nitrogen source and of 1.5% glycerol as constant C source. The overall test conditions were identical to those of earlier series. Initial and final pH values were established; the test duration was held to 14 days. The starting reaction in all tubes corresponded to pH 6.8, i.e. it was only slightly acid.

Tab.12. Pigment formation and cell growth in the presence of various concentrations of NH_4Cl / 1.5% glycerol.

NH_4Cl	factor of multiplication	pigment %	final pH	NH_4Cl	factor of multiplication	pigment %	final pH
0.1	8.0	47.5	5.6	0.7	3.0	2.5	5.4
0.2	8.0	100.0	5.4	0.8	3.0	2.5	5.5
0.3	7.0	100.0	5.4	0.9	2.0	2.5	5.6
0.4	6.0	30.0	5.4	1.0	1.0	5.0	5.7
0.5	5.0	7.5	5.5	2.0	2.0	1.0	5.6
0.6	6.0	50.0	5.5	3.0	1.0	0.5	5.6

Table 12 shows that ammonium chloride is tolerated only in higher dilutions in the presence of glycerol, since the optima of growth and pigmentation occur at an ammonium chloride concentration of 0.2-0.3%. The final pH values are all found in the acid area and show, as could be expected, slightly more acidity at optimal growth than at poor growth, as revealed by the last column in table 12, where the final pH values are listed according to starting concentrations of ammonium chloride.

Test series 5.

In comparison to others, this test series only shows a different nitrogen source; asparagine was used in place of ammonium chloride. All other conditions were the same.

Tab.13. Pigmentation and cell multiplication in the presence of various concentrations of asparagine + 1.5% glycerol.

Asparagine %	Factor of multiplication	pigment %	Final pH	Asparagine %	Factor of multiplication	pigment %	Final pH
0.1	14	12.5	5.8	0.7	4	2.5	5.9
0.2	7	7.5	5.7	0.8	15	2.5	6.7
0.3	25	62.5	6.6	0.9	20	60.0	6.8
0.4	10	2.5	6.0	1.0	17	85.0	6.6
0.5	6	2.5	6.1	2.0	8	100.0	6.6
0.6	3	5.0	6.2	3.0	2	5.0	6.3

The jump at 0.3% asparagine must be attributed to an error in inoculation, since all values increase in unison. Optimal pigment formation lies at a concentration of around 2% asparagine, while optimal growth requires a slightly lower quantity. In the presence of ammonia-yielding asparagine as N source, the highest pH values also correspond to maximal growth, which demonstrates that good reproduction does not shift the reaction to a more acid area, but maintains the solution in a nearly even, buffered state.

VII. Influence of the initial reaction of the medium on growth and chromogenesis.

It has long been known that the initial reaction of the medium affects pigmentation and reproduction of B. prodigiosus. The inhibiting effect of strongly acid or alkaline media has been familiar for some time, and investigators knew that a weakly acid reaction is favorable to pigment production, while multiplication is best on slightly alkaline nutrients. Thus cultures of B. prodigiosus on slightly acid nutrient agar show a deep, dark red pigment with a metallic sheen, while cultures on weakly alkaline agar, although with excellent growth, only produce a pale red pigment. The following tests were designed to uncover the influence of initial pH values on the growth and pigment formation of B. prodigiosus in media of known composition. Nutrient solutions with asparagine or ammonium chloride as nitrogen sources and 3% glycerol as constant carbon source were utilized, the reaction of which was calibrated by the addition of hydrochloric acid or potash lye. In both series cultivation took place at 28°C and lasted 10 days.

Test series 1.

Here asparagine (0.656%) served as N source and glycerol (3%) as C source.

Table 14 shows that under existing conditions, optimal chromogenesis actually takes place in the weakly acid area; the same is true for optimal growth. The column of final pH values reveals that there is a trend to establish a uniform reaction in the area of pH 6-6.3, since the low pH values generally rise and the high ones fall, if the unavoidable fluctuations are disregarded.

Tab.14. Pigmentation and cell multiplication in asparagine-glycerol solution of various reactions.

Initial pH	Factor of multiplication	pigment %	Final pH	Initial pH	Factor of multiplication	pigment %	Final pH
4.6	2.0	0	4.6	6.6	9.0	15.0	6.6
5.0	6.0	32.5	4.6	7.0	6.0	20.0	6.6
5.4	9.0	47.5	6.6	7.3	8.0	5.0	6.3
5.6	13.0	7.5	6.4	7.5	9.0	22.5	6.8
5.8	13.5	9.0	5.9	7.8	7.0	15.0	6.8
6.0	14.0	35.0	6.2	8.0	12.0	5.0	6.7
6.2	13.5	20.0	6.2	8.2	2.0	2.5	6.0
6.4	19.0	100.0	6.7	8.6	3.0	4.0	6.0

Test series 2.

In this test series ammonium chloride in the amount of 0.535 g per 100 ccm nutrient solution was utilized as nitrogen source in place of asparagine. This test had to be limited to the area of pH 5.4-7.2, since stronger alkalinity causes the formation of the poorly soluble compound magnesium-ammonium phosphate from Mg and PO_4 ions present in the nutrient solution, which then precipitates from the alkaline solution. Also, stronger alkalinity causes separation of ammonia from ammonium chloride, which also must be avoided. Otherwise the same conditions and circumstances prevail as in test series 1 of this chapter.

Tab.15. Chromogenesis and growth in ammonium chloride-glycerol solution of various reactions.

Initial pH	Factor of multiplication	pigment %	Final pH	Initial pH	Factor of multiplication	pigment %	Final pH
5.4	1	0	4.9	6.6	4	2.5	5.2
5.6	1	0	5.0	6.8	4	2.5	5.3
6.0	4	2.5	5.0	7.0	7	2.5	5.4
6.2	2	2.5	5.0	7.2	3	20.0	5.4
6.4	3	100.0	5.1				

Table 15 shows that optimal pigment production takes place at a strict pH value of 6.4, i.e. again in the weakly acid area. Growth, on the other hand, increases rather steadily toward the alkaline region and has a slightly increased optimum at pH 7. In comparison to the first test of this chapter nothing has changes regarding optimal chromogenesis. Minimum pigmentation takes place at pH 6 and in the alkaline portion. Maximum pigmentation corresponds to a final pH value of 5.1. Besides, the terminal hydrogen ion concentration has risen to such an extent compared to the initial, that almost equal pH values have developed throughout.

The different test series reveal unequal influence on chromogenesis and a connection between the latter and growth on media with simple, easily defined nitrogen and carbon sources to the extent that a method of cultivation

can be derived therefrom for the purpose of producing corresponding quantities of the pigment of B. prodigiosus, making the investigation of prodigiosin possible. Results of such an investigation will be discussed in a second report.

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